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(54) Title: INDUCTION OF CYTOTOXIC T LYMPHOCYTES (CTL) USING ANTIGENIC PEPTIDES AND A SUITABLE ADJUVANT

(57) Abstract

The prevent invention provides a method of treating a tumor-bearing subject, a method of inducing cytotoxic antitumor T lymphocytes, a method of treating pathogenic disease, and a method of vaccinating a subject comprising administering an MHC Class I restricted, 8-12 amino acid residue antigenic peptide in combination with a suitable adjuvant, such as TiterMax.

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# INDUCTION OF CYTOTOXIC T LYMPHOCYTES (CTL) USING ANTIGENIC PEPTIDES AND A SUITABLE ADJUVANT

Throughout this application, various publications are referenced by arabic numerals in brackets. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

## BACKGROUND OF INVENTION

$CD8^+$ major histocompatibility complex (MHC) class I molecule - restricted cytotoxic T lymphocytes (CTL) play a major role in mounting a specific immune response against intracellular pathogens. A successful strategy to induce specific CTL responses against these pathogens would greatly enhance the ability to control various viral, parasitic and bacterial diseases by vaccination. Recently, several groups reported successful induction of CTLs using multiple doses of 15-16 amino acid long synthetic peptides emulsified in the incomplete Freund's adjuvant. However, general applicability of this method was not tested.

This invention discloses a simple and highly reproducible method of generating CTL activity against eight tested CTL determinants by a single dose of peptide immunization, in two different strains of mice. The immunization strategy combines the optimal MHC class I restricted peptide determinant (octa- or nonameric peptide) with a synthetic, commercially available adjuvant TiterMax®. CTL activity elicited in this fashion is mediated by $CD8^+$ cells, and is physiologically relevant: peptide-elicited CTLs are capable of lysing

target cells that endogenously synthesize and process the determinant, including virally infected targets. The advantages of this method over previously published methods are that : **(i)** 8-10 amino acid long peptides, optimal for class I binding, are very effective; **(ii)** a single injection is sufficient to induce a strong response; and **(iii)** the method is generally applicable, since it produced results with six H-$2^b$ restricted peptides and two H-$2^d$ restricted peptides. Because of the small peptide size, the chance of autoimmune and allergic complications is greatly reduced and the cost is also reduced.

Most importantly, this method can be used to elicit potent anti-tumor CTLs. In fact, in experiments, peptide immunization induced CTLs that protect animals against otherwise lethal tumors. Thus, peptide treatment and vaccination has considerable diagnostic, preventive and therapeutic potential.

## SUMMARY OF INVENTION

The present invention provides a method of treating a subject with a tumor which comprises administering to the subject an effective amount of a MHC Class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby treat the subject with the tumor. Further, the suitable adjuvant is TiterMax®.

In addition, the present invention provides a method of inducing cytotoxic T lymphocytes in a subject which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby induce anti-tumor cytotoxic T lymphocytes in the subject. Further, the present invention provides for an anti-tumor cytotoxic T lymphocyte.

In addition, the present invention provides a method of treating a subject with a pathogenic disease which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby treat the subject with the pathogenic disease.

In addition, the present invention provides a method of vaccinating a subject which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby vaccinate the subject.

## BRIEF DESCRIPTION OF FIGURES

**Figure 1A-1B.** Surface phenotype of peptide-induced CTLs. HSV peptide-induced CTL lines were phenotyped for the expression of $CD8^+$ and $CD4^+$ (Figure 1A) and TCR (Figure 1B) as described in Methods, and expressed as contour plots (Figure 1A) or open histograms (Figure 1B). Filled histogram in (Figure 1B) represents the fluorescence of control-stained cells. Similar results were obtained with CTL lines induced by five different peptides.

**Figure 2.** CTL activity of spleen cells obtained by immunization with HSV pep/TM, TM or pep. Immunization, restimulation and CTL assay were performed as described in Methods. CTL activity was tested on HSV peptide - coated EL-4 cells or control EL-4 cells. The lysis of the latter was <5%.

**Figure 3.** $K^d$-restricted CTLs induced by pep/TM. B6D2 $F_1$ mice were immunized by SVT/TM (positive control), LLO/TM or p60/TM, restimulated in vitro, and tested for cytolytic activity against EL-4 or P815 cells in the presence (solid symbols) or the absence (open symbols) of corresponding peptides. Results from primary cultures are displayed.

**Figure 4A.** E.G7 tumor is not rejected by lightly irradiated animals. B6 mice (2/group) were irradiated with 4 Gy and inoculated subcutaneously in the flank with either 2 x $10^7$ (triangles) or 2 x $10^6$ (circles) E.G7

cells. Tumor growth and status was monitored daily, and is expressed as tumor diameter (mm), obtained by multiplying two orthogonal measurements of the tumor using calipers, and by extracting a square root from this value.

__Figure 4B__. Vaccination with pep/TM protects against tumor growth. B6 animals were immunized with OVA/TM on day 0. Seven days latter, mice were irradiated and injected with E.G7 cells (7 x $10^6$/recipient). Tumor growth was scored as described in Figure 4A.

__Figure 5__. Cocktail immunization can elicit CTL activity against each of the peptides in the cocktail mixture. B6 mice were immunized with a cocktail containing 5$\mu$g each of OVA, FLU and SVT peptides in TM. CTL activity of split cultures restimulated on each of the three peptides is shown on EL-4 targets with (filled symbols) and without (open symbols) peptide. Representative results from three experiments are shown. FLU-triangles; OVA-circles; SVT-diamonds.

__Figure 6.__ $CD4^+$ dependence of pep/TM responses. B6 mice were treated with successive injections of purified GK 1.5 mAb (circles) or saline (triangles), and immunized with the SVT peptide. CTL activity was tested after __in vitro__ restimulation against EL-4 target cells in the presence (filled symbols) or absence (open symbols) of the SVT peptide.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of treating a subject with a tumor which comprises administering to the subject an effective amount of a MHC Class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby treat the subject with the tumor.

In one embodiment the amino acid antigenic peptide is a small peptide sequence. In another embodiment the amino acid antigenic sequence is no more than 15 amino acids. In the preferred embodiment the amino acid sequence is 8-10 amino acids.

The "MHC class I restricted 8-12 amino acid antigenic peptide" is defined herein as a 8-12 amino acid sequence which when administered to the subject induces cytotoxic T lymphocytes (CTL) via MHC class I molecules in combination with a suitable adjuvant.

In one embodiment the suitable adjuvant consists of, or is a combination with, a metabolizable oil, squalene, or a block copolymer. In the preferred embodiment the suitable adjuvant is TiterMax® or "TITERMAX" (Vaxcel™, Inc,). More specifically, TiterMax® consists of a block copolymer CRL-8941, microparticular silicia coated with CRL-8941, sorbitan monooleate 80 and squalene.

Further, the effective amount the MHC class I restricted 8-12 amino acid antigenic peptide in combination with the effective amount of a suitable adjuvant is administered in combination with a second anti-tumor therapy.

As defined herein "a second anti-tumor therapy" is any therapy which is employed to treat a subject with a tumor. For example, therapies include but are not

limited to: irradiation, cytostatic or chemotherapy. Chemotherapeutic agents, include but are not limited to: alkylating agents, i.e. nitrogen mustards, ethylenimines and methylemelamines, alkyl sulfonates, nitrosoureas, and triazenes. Further chemotherapeutic agents include antimetabolites, i.e. folic acid analogs, pyrimidine analogs, purine analogs and related inhibitors.

Further chemotherapeutic agents include natural products, i.e. vinca alkaloids, epipodophyllotoxins, antibiotics, enzymes, biological response modifiers. Further, chemotherapeutic agents include miscellaneous Agents, i.e. platinum coordination complexes, anthracenedione, substituted urea, methyl hydrazine derivative, and adrenocortical suppressant. Lastly, chemotherapeutic agents include hormones and antagonists, i.e. adrenocorticosteroids, progestins, estrogens, antiestrogen, androgens, antiandrogen, and gonadotropin-releasing hormone analog.

As defined herein "tumor" includes but is not limited to: sarcomas, carcinomas, fibrosarcoma, osteocarcoma, chondrosarcoma, neuroblastoma, retinoblastoma, B cell lymphoma, myeloblastic leukemia, and lymphatic leukemia.

Further, it is well known is the art how to determine the antigen or epitope of the CTL for a given tumor and the effective amount of the MHC Class I restricted 8-12 amino acid antigenic peptide corresponding to the tumor which is administered to the subject. Synthetically generating the peptide for the purpose of inducing and testing CTL responses are known to one skilled in the art [4]. For example, the HLA-B18 and HLA-B35 restricted epitope from the CMV may be employed.

Further, taking advantage of a high level bacterial expression vector, the regularity of exonuclease III DNA

degradation, and rapid alkali hydrolysis, the CTL antigen can be located within cloned genes. Exonuclease III degrades DNA at roughly 200 nucleotides/min and only from a blunt or 5' overhang terminal. Thus, a large panel of tightly nested deletions in the 3'- end of a gene may be constructed within an inducible prokaryotic expression vector. After IPTG-mediated induction of transcription, vector, derived protein is expressed at high levels for several hours and eventually constitutes approximately one-third of the protein in these bacteria. This is adequate purity for the generation of targeting peptides from alkali digests of whole or lysed bacteria [3].

Further, E. coli over-expressing the cancer cell protein of interest of lysates derived from such cultures may also be digested by alkali hydrolysis to generate targeting peptide. The location of the epitope may then be determined from a panel of Escherichia coli clones expressing various 3'- truncated forms of the gene. The general applicability of this approach was demonstrated by screening two genes from the common pathogen, human CMV for two HLA class I-restricted epitopes. This method requires limited information about the target Antigen and the restricting MHC to rapidly and precisely localize CTL epitopes [4].

For example, the antigenic peptides for MAGE which are present in melonoma, breast and bladder cancer are: EVDPIGHLY, EADPTGHSY, EVVPISHLY (SEQ. ID. NOS. 1-3). Further, specific antigenic peptides for tumor cells are known to one skilled in the art.

In addition, this invention provides a method of inducing cytotoxic T lymphocytes in a subject which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of "TITERMAX" so as

to thereby induce cytotoxic T lymphocytes in the subject.

Further, the cytotoxic T lymphocytes is a anti-tumor cytotoxic T lymphocytes. As defined herein "anti-tumor cytotoxic T lymphocytes" are CTL's specifically induced by an antigen, which is associated with a tumor or is present on tumor cells.

Further, the method of treating a subject with a tumor may be with a plurality of antigenic peptides which are administered in combination with an effective amount of the suitable adjuvant.

In addition, this invention provides a method of treating a subject with a pathogenic disease which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby treat the subject with the pathogenic disease.

In one embodiment the pathogenic disease is bacterial. In a another embodiment the pathogenic disease is parasitic. In another embodiment the pathogenic disease is viral.

Further, bacterial diseases include, but are not limited to: Gram negative bacilli, such as Salmonella; Spirochetes; Gram positive cocci, such as, Staphylococcus aureus, Streptococcus; Gram negative cocci, such as Neisseria gonorrhoea; Gram positive bacilli, such as Escherichia coli; and Gram negative bacilli; Acid fast bacilli.

Further, parasitic diseases include, but are not limited to: protozoan infections, such as Leishmaniasis, Trichomoniasis, Trypanosomiasis, Malaria, Amebiasis,

Balantidiasis, and Giardiasis; and metazoan infections, such as, Hookworm, Trichinosis.

Further, viral diseases include, but are not limited to: Human Immunodeficiency Virus, Herpesvirus, Varicella-Zoster Virus, Cytomegalovirus, Epstein-Barr Virus, Hepatitis B, Papillomavirus, Influenza, and Respiratory Synctial Virus, and Simian Virus 40 (SVT).

Further, the present invention includes, but is not limited to, MHC class I restricted 8-12 amino acid antigenic peptides consisting of: EVDPIGHLY, EADPTGHSY, EVVPISHLY, EIRSLYNPV, PLTSCNTSV, GYKDGNEYI, KYGVSVQDI, SIINFEKL, RGYVYQGL, FAPGNYPAL, VVYDFLKCL, SSIEFARL, ASNENMETM, GILGFVFPL, LLFGYPVYV, ILKEPVHGV, KLGEFYNQMM, IAGNSAYEYV, FLASDFFPSV (SEQ. ID. NOS. 1-19).

Further, this invention provides for a plurality of antigenic peptides which can be administered in combination with an effective amount of the suitable adjuvant.

In addition, this invention provides a method of vaccinating a subject which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby vaccinate the subject.

As defined in this invention, the word "vaccine" is an antigen source for activating an immune responses against established tumors or pathogenic diseases, and thus for prophylactic and preventative immunization.

Further, the method of vaccinating a subject may be with a plurality of antigenic peptides which are administered in combination with an effective amount of the suitable

adjuvant as hereinabove described.

In addition, the present invention provides, a method of treating a subject with an auto-immune disease, which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide, in combination with an effective amount of a suitable adjuvant, so as to thereby treat the subject with the auto-immune disease.

As defined herein "auto-immune" diseases include, but are not limited to: Hashimoto's Thyroiditis, Pernicious Anemia, Addison's disease, Goodpasture's syndrome, male infertility, Multiple Sclerosis, Idiopathic leucopenia, Ulcerative colitis, Rheumatoid arthritis, Scleroderma, Systemic Lupus Erythematosus. Other immune disorders include graft vs. host rejection and immunoincompetent subjects.

In addition, the present invention provides a method of assaying the MHC class I restricted antigenic peptide of a tumor of a subject by contacting the tumor of a subject with a panel of cytotoxic T lymphocytes with known MHC class I restricted 8-12 antigenic peptides so as to assay the MHC class I restricted antigenic peptide of a tumor.

In addition, the present invention provides, a method of inducing cytotoxic T lymphocytes in a subject which comprises administering to the subject an effective amount of a MHC class I restricted amino acid antigenic peptide in combination with an effective amount of "TITERMAX" so as to thereby induce cytotoxic T lymphocytes in the subject.

Further, the method of inducing cytotoxic T lymphocytes in a subject may be with a plurality of antigenic peptides which are administered in combination with an

effective amount of the suitable adjuvant. Further, the antigenic peptides have hereinabove been described.

In addition, this invention provides, a kit for inducing cytotoxic T lymphocytes in a subject which comprises a suitable amount of MHC class I restricted 8-12 amino acid antigenic peptide and a suitable adjuvant. Further, the suitable adjuvant is "TITERMAX". Further, the antigenic peptides have been hereinabove described.

The kit may include, but is not limited to: MHC class I restricted 8-12 amino acid antigenic peptide in saline or other suitable fluid as hereinabove described (50 ng - 50 μg); a block copolymer, such as CRL89-41 bonded to a the surface of a silica particles; water-in-oil emulsion containing a metabolizable non-toxic oil; squalene; and plastic syringes or other means to prepare suitable adjuvant for emulsification. Further, reagents include but are not limited to, demulsifying agents, such as SDS or other acrylamide gels.

As defined herein the "subject" may be a human, monkey, dog, cat, rabbit, horse, cow, chicken and rodent. In the preferred embodiment the subject is a human. In another embodiment, the subject is a rodent, more specifically a mouse.

As used herein administration means a method of administering to a subject. Such methods are well known to those skilled in the art and include, but are not limited to, administration topically, parenterally, orally, intravenously, intradermally, intranasally, intratumorally, intratracheal, intramuscularly, subcutaneously, or by catheter. Administration of the agent may be effected continuously or intermittently such that the therapeutic agent in the subject is effective to modulate or treat the neoplastic cell or tissue.

Further, in the present invention booster shots, which are defined as shots after the initial administration, are not required. However, suitable regimes for initial administration and booster shots are variable, but are typified by an initial administration followed by repeated doses at one or more hour or day intervals by a subsequent injection or other administration may be employed.

In addition, this invention provides a MHC Class I restricted 8-12 amino acid antigenic peptide in combination with a suitable adjuvant which may be formulated into the therapeutic composition so as to be neutralized pharmaceutically in an acceptable salt forms.

As defined herein "effective amount" is in a range of about 50 ng to 10 mg. In one embodiment the effective amount may be up to 1 g. In another embodiment the effective amount is from about 2 to 50 $\mu g$, more preferably the effective amount is 5 $\mu g$. In a preferred embodiment, 5 $\mu g$ of peptide and 10 $\mu L$ of a suitable adjuvant are administered in a single injection. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual.

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

## EXPERIMENTAL DETAILS

### Materials and Methods:

***Mice.*** C57BL/6 (B6, H-$2^b$), B6D2 $F_1$ (H-$2^{b \times d}$), and BALB/c (H-$2^d$) mice were obtained from the National Cancer Institute animal facility (Frederick, MD). B10.D2 (H-$2^d$) animals were purchased from Jackson Laboratories (Bar Harbor, ME). Animals of both sexes were used at 6-10 weeks of age.

***Peptides.*** Peptides were synthesized by the Memorial Sloan-Kettering Cancer Center (MSKCC) Microchemistry Core Facility using a standard f-moc method, followed by HPLC purification and mass spectroscopy analysis, and were >98% pure, as evaluated by these methods. Sequences of peptides used in the study are shown in Table II.

***Cell lines and viruses.*** MHC class II negative cell lines EL-4 (H-$2^b$), its variants transfected with ovalbumin [18] and VSV nucleoprotein [12], P815 (H-$2^d$) and its variant transfected with listeriolysin O [11], and MC57 and its variant transfected with the Herpes Simplex virus glycoprotein B [19] were grown in RP10 [RPMI 1640 supplemented with antibiotics, 2-ME, glutamine, HEPES and 10% FBS (Gemini Bioproducts)]. Influenza virus strain A/PR8/34 (H1N1) [PR8 in the text], and VSV strain Indiana were obtained from Dr. M.J. Bevan (Univ. of Washington, Seattle, WA). HSV type I strain 17 was generously provided by Dr. S. Silverstein (Columbia University, New York, NY). Indicated cell lines were infected with viruses in serum-free medium (RPMI 1640) at : (i) 10 pfu (plaque forming units)/cell for 1 h at 37°C for HSV, followed washing and a 5 hr incubation at 37°C; or (ii) with $10^{-4}$ HAU (hemaglutininn units) for 90′ for PR8; or (iii) with 50 pfu VSV/cell, for 90′. For HSV infection, infected cells were then washed and labeled with $^{51}$Cr

before the CTL assay. For PR8 and VSV, chromium labeling was performed simultaneously with infection. In vivo immunization with viruses was carried out by a single i.p. injection of $10^6$ pfu/animal (VSV and HSV) or 300 HAU (influenza).

***Immunization***. Details of immunization protocols other than the one using Titermax® were described in references [1,5,9,10,13,14]. For peptide/Titermax® (pep/TM) immunizations, peptides were emulsified in Titermax® according to manufacturer's instructions, in the concentration of 0.5 mg/ml, and 10 $\mu$l of this mixture injected s.c. into one footpad of methophane-anesthetized animals (unless otherwise indicated). This immunization did not appear to cause pain, discomfort, local inflamatory or allergic reactions. Non-anesthetized mice were also immunized without aberrant pain, stress, or discomfort [16].

***Cytotoxic T Lymphocyte restimulation and $^{51}$Cr-release assay***. Six to eight days after restimulation, spleen cells from immunized animals were restimulated in 25-cm tissue culture flasks (Falcon, Becton-Dickinson, Mountain Viwe, CA), at 2-3 x $10^7$ cells/flask in the presence of 2 x $10^7$ peptide-coated irradiated (30 Gy) syngeneic spleen cells. Peptide coating was performed with 100 $\mu$g of peptide/spleen, in HBSS without FBS, for 1h at 37°C, followed by three washes. Cultures were incubated at 37°C/5%$CO_2$, in a total volume of 10 ml of RPMI 1640 / 10% FCS (RP 10), with other supplements as described [10]. Cytotoxic activity of restimulated cultures was determined after five days in a classical 3-4 h $^{51}$Cr-release assay. Target labeling, peptide coating and the assay were performed exactly as described [10], except that the standard peptide concentration for coating was 2 $\mu$g/ml (unless indicated otherwise).

***Flow cytometric (FCM) analysis.*** Phenotype of cultured CTLs was determined using anti-CD8-FITC, anti-CD4-PE and anti-TcR$\beta$-FITC antibodies purchased from PharMingen (San Diego, CA). $10^6$ cells were simultaneously stained and analyzed for the expression of $CD4^+$ and $CD8^+$, or singly stained with anti-TCR$\beta$. After washing, cells were analyzed using a FACScan instrument and the LYSYS II software (Becton-Dickinson, Mountain View, CA). Control samples were stained with conjugated, species- and class-matched irrelevant antibodies (Fisher Biotech, Malvern, PA). Results from $10^4$ cells/sample are displayed as contour plots.

***Antitumor activity of pep/TM-induced CTLs.*** For this assay, immunized or naive mice were irradiated with 4 Gy, anesthetized with methophane, their left flank shaven, and tumor injected s.c.. Mice were monitored daily for the tumor formation and the size of tumor measured by calipers. Mice were euthanized as soon as any of the following conditions were fulfilled: loss of motility, tumor ulceration, necrosis or inflamation, or tumor size of 25 mm.

**Experiment 1. Induction of CTL Activity by Peptide Priming.**

Recently, several groups induced CTL responses against purified or recombinant proteins of intracellular pathogens. These authors used vaccinia vectors [2] immunostimulatory complexes containing detergent components [18] or physical methods (electroporation) to deliver complex protein antigens into the cytosolic processing pathway [5], often by using several booster injections of the antigenic mixture. However, the production of such complex vaccines is usually elaborate, and the treatment could be complicated by multiple injections and the antigenicity of the whole protein.

A preferable strategy would be to use smaller fragments of proteins to elicit CTL immunity, but the delivery of such fragments to class I molecules poses a problem. Indeed, CTL priming was recently achieved by peptides conjugated to lipid moieties [15] or emulsified in adjuvants [1] and in two instances it was shown that such immunization and subsequent boosting elicits CTLs that can protect against viral infection [7,8,17]. Peptides used for immunization were usually 15-16 residues long, and often contained not only a CTL but also a helper T lymphocyte (HTL) determinant. However, none of these protocols were tested with more than a single peptide and with more than one restricting MHC class I molecule. Potential advantages of shorter peptides would be : **(i)** simpler and cheaper synthesis; **(ii)** a lower probability to elicit unwanted (e.g. autoimmune) consequences; and **(iii)** an independence of their MHC class I binding on additional proteolytic processing.

In an attempt to elicit specific CTL responses in H-2K$^b$ animals against the optimal ovalbumin peptide 257-264, SIINFEKL (OVA) (SEQ. ID. NO. 8), or VSV nucleoprotein peptide 52-59, RGYVYQGL (SEQ. ID. NO. 9) several protocols of immunization were tested in the same experiment. The only three priming protocols that yielded significant CTL activity specific for peptides were: thioglycolate-induced peritoneal macrophages coated with OVA, and peptide mixed with $\beta_2$-microglobulin ($\beta_2$-m), both as described by Rock et al. [13,14] and peptides emulsified in the synthetic adjuvant TiterMax®, abbreviated in the text as pep/TM (Table I). In subsequent experiments only pep/TM yielded reproducible results. Therefore CTL response to peptides in adjuvant was characterized, and tested to a broad range of peptides in order to evaluate the general features of this system.

Initially priming was achieved with a single subcutaneous injection of a very low peptide amount (5 μg). CTL response was vigorous and similar to that obtained in animals immunized with transfectants expressing the OVA determinant (Table I), or with spleen cells cytoplasmically "loaded" with the whole ovalbumin protein [9], two strategies commonly used to immunize with endogenously processed OVA. CTLs induced in this manner were propagated like conventional CTLs in a continuous culture by weekly restimulations with antigen and IL-2-rich Con A Sn; they were of $CD8^+4^-$ $TcR\alpha\beta^+$ phenotype (Figure 1A and Figure 1B). Most importantly, the method was highly reproducible: CTLs were elicited in every single immunized B6 mouse, as shown in Table II (individual mice, and not pooled spleen cells were tested).

TABLE I. Methods of <u>in vivo</u> CTL priming by peptides

| **Immunization Protocol** | | **Animals responding** | **%$^{51}$Cr release** |
|---|---|---|---|
| E.G7 cells s.c. | | 2/2 | 74%,49% |
| Soluble protein | i.v. | 0/2 | NA |
| Soluble protein/ 150 mM NaCl s.c | | 0/2 | NA |
| Soluble peptide | i.v. | 0/2 | NA |
| Soluble peptide | s.c. | 0/2 | NA |
| Peptide/IFA | s.c. | 0/2 | NA |
| Peptide/CFA | s.c. | 0/2 | NA |
| Peptide/spleen | i.v. | 0/2 | NA |
| Peptide/spleen, | s.c | 0/2 | NA |
| Peptide/RMA-S | s.c. | 0/2 | NA |
| Peptide/TM | s.c. | 2/2 | 68%;67% |
| Peptide/TM | i.p. | 1/2 | 71% |
| Peptide/$\beta_2$-m | s.c. | 1/2 | 70% |
| Peptide/Mρ | i.v. | 1/2 | 40% |
| Peptide/Mρ | s.c. | 0/2 | NA |

For Table 1. immunizations were performed in a single injection and at a single site. Wherever soluble peptide or peptide in adjuvant was used, the concentration of the peptide was 5 $\mu$g/injection, in a total volume of 10 $\mu$l. Emulsification in adjuvants was performed according to manufacturers' specifications. Coating of spleen cells, elicited macrophages (M$\rho$), and RMA-S tumor cell lines was performed using 100 $\mu$g of peptide and $10^8$ cells for 1 h at 37°C, followed by three washes. $\beta_2$-m was used at 10 $\mu$g/injection, as described [13]. Seven days after immunization, splenocytes were restimulated with irradiated B6 spleen cells and EL-4 transfectants expressing endogenously processed OVA or VSV, in RPMI medium supplemented as described [10]. On day 5 of restimulation, cultures were tested for the presence of anti-OVA CTL activity using peptide-coated, $^{51}$Cr-labeled EL-4 transfectant cells E.G7 (OVA) and N1 (VSV nucleoprotein), both H-$2^b$, class II$^-$, as targets. Number of responding animals that displayed more than 20% transfectant-specific lysis is expressed as a fraction of total number of animals tested. %$^{51}$Cr-release is displayed for responding animals. Lysis of control untransfected EL-4 cells was rather high, since these tumor cells were also used for restimulation <u>in vitro</u>, and varied from 10 to 35%.

**Experiment 2. Peptide Priming as a General Method to Elicit Physiologically Relevant CTLs**

To investigate whether this priming protocol may be effective for other peptides. Mice were immunized with four other $K^b$-restricted peptides, and one $D^b$-restricted peptide. Table III summarizes the obtained results. In every case potent CTL activity, specific for the immunizing, but not for irrelevant peptides, was obtained in each animal. The only exception was the H-$2D^b$-

restricted influenza nucleoprotein peptide 366-374 (FLU-$D^b$), where three out of four animals mounted a good response, but the fourth responded weakly (~25% specific lysis). However, that line showed a substantial improvement of CTL activity upon the second <u>in vitro</u> restimulation. This subpar primary response could have been caused by a loss of priming mixture from the footpad due to leaking. An example of actual lysis by primary CTL cells is shown in Figure 2. Adjuvant alone did not elicit any CTL activity, whereas soluble peptide yielded low specific lysis, and CTLs from these cultures could not be propagated further (Figure 2).

CTLs against peptides were obtained previously, but were: **(i)** of low affinity, since they required high micromolar concentrations of antigen for target sensitization; and **(ii)** were specific for contaminating products in the peptide preparation, and not for the physiologically relevant peptides derived by endogenous processing and presentation [16]. Such CTLs were actually induced by peptide <u>in vitro</u>. By contrast, results shown in Table III. indicated that CTLs typically lysed target cells coated with as little as 10-100 pM of peptide. To test the specificity of CTL lines derived by peptide priming <u>in vivo</u>, ability of peptide-primed CTLs to lyse cell lines transfected with proteins from which the peptides were derived were examined (Table IV). CTL lines induced by pep/TM specifically lysed such transfectants, clearly showing that they are specific for physiologically processed peptides.

B6D2 $F_1$ mice were immunized with two optimal peptides derived from two different proteins of *L. monocytogenes*, [the major determinant of listeriolysin O (GYKDGNEYI (SEQ. ID. NO. 6) [5]) and the peptide derived from the secreted, invasion-related protein p60 (KYGVSVQDI) (SEQ. ID. NO. 7), both restricted by $H\text{-}2K^d$. CTL activity

obtained from these mice was strong and comparable to that obtained with H-$2^b$ - restricted peptides (Figure 3). It was concluded that pep/TM method can be universally used with any MHC molecule and its corresponding optimal determinant(s).

**TABLE II. Peptide-primed CTLs can be induced by a variety of viral peptides (SEQ. ID. NOS. 8-13)**

| Peptide | Origin | Sequence | # of responding animals/total animals immunized |
|---|---|---|---|
| OVA | Ovalbumin | SIINFEKL | 13/13 |
| VSV | VSV nucleoprotein | RGYVYQGL | 16/16 |
| SEN | Sendai nucleoprotein | FAPGNYPAL | 2/2 |
| SVT | SV40 large T | VVYDFLKCL | 6/6 |
| HSV | Herpes simplex gB | SSIEFARL | 11/11 |
| FLU-$D^b$ | Influenza nucleoprotein | ASNENMETM | 4*/4 |

For Table II. B6 mice were primed by a single footpad injection of 5 $\mu$g of indicated peptide in 10 $\mu$l of Titermax, and CTL activity assayed on peptide-coated EL-4 cells as described in the legend to Table I. Spontaneous lysis was < 20% of maximal, and all animals displayed specific lysis levels between 40 and 70%. Lysis of EL-4 cells in the absence of peptide was <5%.

**TABLE III. Peptide-induced CTLs recognize target cells sensitiz d with picogram concentrations of peptide**

| **CTL** specificity | **Concentration of SEV peptide ($ml^{-1}$) used to coat EL-4 targets** 5μg | 50 ng | 500 pg | 5 pg | 50 fg | no peptide |
|---|---|---|---|---|---|---|
| SEV | 68.3 | 69.7 | 72.1 | 56.4 | 4.9 | 4.7 |

For Table III. SEV-specific line LAG was derived by peptide priming and was maintained in culture by weekly restimulations as described. The line was tested against EL-4 cells coated with no peptide or with indicated concentrations of SEV peptide, and the results displayed as % specific $^{51}$Cr-release at the effector:target ratio of 25:1.

**Experiment 3.** ***Dose Dependency of the Priming***

Efficient and reproducible priming with 5 μg of peptide in a single injection was obtained. To establish the limits of sensitivity of this priming protocol, priming dose was varied over several orders of magnitude for three peptides, OVA, VSV and FLU-$D^b$. Results of this assay are summarized in Table V. CTL αOVA response could be elicited by priming with as little as 0.5 μg/animal, although perhaps not as reproducibly as with higher doses. Anti-VSV response displayed similar dose dependency. However, FLU-$D^b$ was effective only at a narrow range of concentrations, with optimal activity at 5 μg. Experiments with other peptides are in progress.

**TABLE IV. Peptide-primed CTL are specific for endogenously processed antigens**

| **Priming peptide** | % specific $^{51}$Cr-release from targets | | | | | |
|---|---|---|---|---|---|---|
| | **EL-4** | **E.G7** | **N1** | **1308.1** | **MC57** | **MC57-gB** |
| TM alone | ND | ND | ND | ND | 4.2 | 6.7 |
| HSV | ND | ND | ND | ND | 5.1 | 79.9 |
| VSV | 0.6 | 1.1 | 80.0 | ND | ND | ND |
| SVT | 4.0 | ND | ND | 28.0 | ND | ND |

For Table IV. peptide priming was performed as described above (Table. II) and CTL activity of anti-peptide cell lines tested on transfectants expressing endogenously processed peptide determinants. E.G7 and N1 are an EL-4 transfectants with ovalbumin and VSV nucleoprotein genes, respectively. 1308.1 is a H-2$^b$ thymic epithelioma expressing the SV40 large T antigen, MC57 is a H-2$^b$ fibrosarcoma, while MC57-gB has been transfected with the glycoprotein B of HSV.

**TABLE V. Dose dependency of peptide priming**

| **Priming peptide** | # of responders/total after priming with | | | | |
|---|---|---|---|---|---|
| | 50 μg | 5 μg | 500ng | 50 ng | 5ng |
| OVA | 3/3 | 3/3 | 2/3 | 0/3 | 0/3 |
| VSV | 3/3 | 3/3 | 3/3 | 0/3 | 0/3 |
| FLU-D$^b$ | 0/2 | 2/2 | 0/2 | 0/2 | 0/2 |

For Table V. B6 mice were primed with indicated peptides in Titermax, and CTL activity of spleen cultures assayed after *in vitro* restimulation as described above. CTL activity higher than 25% of specific $^{51}$Cr-release for VSV and higher than 40% for OVA and FLU-D$^b$ was considered

specific. This cutoff was confirmed by in vitro secondary stimulation of borderline cultures (those exhibiting 12-18% specific lysis), none of which gave any specific lysis upon re-testing. Lysis of EL-4 in the absence of peptides did not exceed 6% in any group.

**Experiment 4. In vitro Antiviral Activity of Peptide-Induced CTLs**

To test whether peptide-primed CTLs could be used to lyse target cells infected with intracellular pathogens, H-$2^b$ cell lines with different viruses were infected, and tested against peptide-induced effector cells. For comparison, virus-induced CTLs were included in the assay in the case of HSV. The optimal HSV peptide induced CTLs that readily lysed virus-infected targets, and this antiviral activity was comparable to that of virus-induced CTLs (Table VI). Similar antiviral activity was obtained with FLU-$D^b$ and VSV peptides (Table VI.).

**TABLE VI. Antiviral activity of peptide-induced CTLs**

| Priming method | % specific $^{51}$Cr-release from targets | | | |
|---|---|---|---|---|
| | MC57 | MC57-gB | MC57/virus | |
| HSV Pep/TM | <1 | 74.9 | 48.2 | |
| live HSV virus | <1 | 60.0 | 47.1 | |
| | EL-4 | EL-4/pept | N1 | EL-4/virus |
| VSV pep/TM | 2.4 | 56.1 | 81.2 | 59.3 |

For Table VI. B6 mice were immunized with the HSV glycoprotein B or VSV nucleoprotein peptide/Titermax® s.c., as described, or with $10^6$ pfu of HSV type 1 , strain 17, i.p.. After the establishment of long-term CTL lines by weekly in vitro restimulation (on peptide-coated spleen cell stimulators for peptide-induced CTLs or on

the transfectant in the presence of irradiated feeder cells for virus-induced CTLs), the CTL activity was tested on indicated targets described in legend to Table III. Results are displayed as the % specific $^{51}$Cr-release at effector:target ratio of 10:1.

**Experiment 5. Antitumor CTL Responses Elicited by pep/TM**

To test whether pep/TM strategy could be used to elicit antitumor CTLs, a tumor model was established by injecting E.G7 thymoma cells s.c. into lightly irradiated syngeneic animals. Unirradiated animals reject this tumor in the course of 10-14 days, and allow only relatively small tumors (5-6 mm of diameter) to form at any time between injection and rejection, even when relatively high doses of tumor cells (e.g. $10^7$) are injected (Figure 4A). By contrast, upon light irradiation (4 Gy), mice injected with either $10^6$ or $10^7$ cells formed tumors (the former slightly slower than the latter) that grew over 20 mm in diameter by 14 days. Owing both to the tumor size and appearance (necrosis, ulceration), animals were euthanized at this point.

Very different results were obtained when mice were vaccinated by pep/TM using the OVA peptide, expressed at the surface of E.G7 cells. While control animals and animals receiving TM alone developed advanced tumors by day 7, and had to be euthanized by day 14 due to vigorous tumor growth, two out of three pep/TM vaccinated animals did not develop tumors during that time at all, while the third animal developed a flat infiltration between days 4 and 7, the diameter of which could not be measured. The infiltration disappeared after day 7 (Figure 4B). These results reveal a considerable antitumor potential of the pep/TM method.

**Experiment 6. Multiple Vaccination by pep/TM**

To establish whether a cocktail of different peptides in TM can simultaneously elicit CTLs directed against each peptide in the mixture, mice were immunized with a cocktail containing OVA and SVT peptides (restricted by $K^b$) and a FLU peptide (restricted by $D^b$). Seven days later, spleen cells of immunized animals were divided in three, and restimulated with each of the three immunizing peptides separately. This protocol resulted in generation of strong and specific reactivity against each of the three peptides used in the immunizing cocktail (Figure 5). These results suggest that this method of immunization can be used to prime against several antigenic determinants on the same microorganism/tumor, or to prime simultaneously against several microorganisms/tumors.

**Experiment 7. CTL Response to pep/TM is not Dependent on $CD4^+$ T Cells**

To test whether $CD8^+$ CTL cells were dependent on $CD4^+$ T cell help, animals deficient for class II molecules owing to a targeted disruption of the I-$A_\beta$ gene were immunized. These mice have a tenfold reduction in $CD4^+$ T cell numbers. To our surprise, these mice mounted a CTL response to HSV and SVT peptides indistinguishable from that of control B6 mice. Given that these mice still contain 5-10% of the normal $CD4^+$ cell numbers, it was still possible that these cells were essential for the CTL response. Therefore $CD4^+$ cells from normal mice were depleted by <u>in vivo</u> mAb treatment (GK 1.5 mAb, 50 $\mu$l of purified mAb on days -1, 2 and 5). Animals treated in this way contained less than 0.5% $CD4^+$ cells in the peripheral blood at the time of immunization and in the spleen at the time of sacrifice. However, CTL responses were intact in these animals (Figure 6). These results

indicate that pep/TM induces $CD4^+$-independent CTLs, and indicate that this method could be successfully used in situations where $CD4^+$ T cells are immunocompromised or scarce, such is the case with *HIV* infections.

**Discussion:**

By using peptides to elicit CTLs one can vaccinate mammals against all three classes of intracellular microorganisms (viruses, bacteria and parasites), and, as demonstrated here, against malignant tumors. A prerequisite for the successful application of this method is the knowledge of the relevant peptide. Several methods can be used to identify such peptides as immunodominant CTL determinants [3,4] and it is possible to identify these peptides out of tumor cells or infected cells in humans [4]. Once the peptides are identified, they can be mixed with a suitable adjuvant, and used to vaccinate animals and humans. Therefore, peptide vaccination should provide a simple general method of eliciting CTL immunity.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Nikolic-Zugic, Janko
Dyall, Rubendra

(ii) TITLE OF INVENTION: INDUCTION OF CYTOTOXIC T LYMPHOCYTES (CTL)USING ANTIGENIC PEPTIDES AND A SUITABLE ADJUVANT

(iii) NUMBER OF SEQUENCES: 19

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(F) ZIP: 10036

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.24

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 08/233,496
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

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(B) TYPE: amino acids
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

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(2) INFORMATION FOR SEQ ID NO:3:

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(B) TYPE: amino acids
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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1               5
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acids
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Glu Ile Arg Ser Leu Tyr Asn Pro Val
1               5
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(2) INFORMATION FOR SEQ ID NO:5:

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(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Pro Leu Thr Ser Cys Asn Thr Ser Val
1 5

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

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1 5

(2) INFORMATION FOR SEQ ID NO:8:

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(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser Ile Ile Asn Phe Glu Lys Leu
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

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(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

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(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

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(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

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(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

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(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

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(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

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(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: N

(vi) ANTI-SENSE: N

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1               5                   10
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What is claimed is:

1. A method of treating a subject with a tumor which comprises administering to the subject an effective amount of a MHC Class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby treat the subject with the tumor.

2. The method of claim 1, wherein the suitable adjuvant is "TITERMAX".

3. The method of claim 1, wherein the effective amount the MHC class I restricted 8-12 amino acid antigenic peptide in combination with the effective amount of a suitable adjuvant is administered in combination with a second anti-tumor therapy.

4. A method of inducing cytotoxic T lymphocytes in a subject which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby induce cytotoxic T lymphocytes in the subject.

5. The method of claim 4, wherein the cytotoxic T lymphocyte is an anti-tumor cytotoxic T lymphocyte.

6. The method of claim 4, wherein the suitable adjuvant is "TITERMAX".

7. A method of treating a subject with a pathogenic disease which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to

thereby treat the subject with the pathogenic disease.

8. The method of claim 7, wherein the suitable adjuvant is "TITERMAX".

9. The method of claim 7, wherein the pathogenic disease is bacterial.

10. The method of claim 7, wherein the pathogenic disease is parasitic.

11. The method of claim 7, wherein the MHC class I restricted 8-12 amino acid antigenic peptide is selected from the group consisting of EVDPIGHLY, EADPTGHSY, EVVPISHLY, EIRSLYNPV, PLTSCNTSV, GYKDGNEYI, KYGVSVQDI, SIINFEKL, RGYVYQGL, FAPGNYPAL, VVYDFLKCL, SSIEFARL, ASNENMETM, GILGFVFPL, LLFGYPVYV, ILKEPVHGV, KLGEFYNQMM, IAGNSAYEYV, FLASDFFPSV (SEQ. ID. NOS. 1-19).

12. A method of vaccinating a subject which comprises administering to the subject an effective amount of a MHC class I restricted 8-12 amino acid antigenic peptide in combination with an effective amount of a suitable adjuvant so as to thereby vaccinate the subject.

13. A method of inducing cytotoxic T lymphocytes in a subject which comprises administering to the subject an effective amount of a MHC class I restricted amino acid antigenic peptide in combination with an effective amount of "TITERMAX" so as to thereby induce cytotoxic T lymphocytes in the subject.

14. The method of claim 1 or 7, wherein a plurality of antigenic peptides are administered in combination

with an effective amount of the suitable adjuvant.

15. The method of claim 1 or 7, wherein the subject is a mammal.

16. The method of claim 15, wherein the mammal is selected from a group consisting of a human, monkey, dog, cow, horse, chicken or rodent.

17. The method of claim 1 or 7, wherein the administration is intratumorally, intradermal, topical, oral, intravenous, intramuscular, intratracheal, or by subcutaneous administration.

18. The method of claim 1 or 7, wherein the effective amount is a range of about 500 ng to about 10 mg.

19. The method of claim 18, wherein the effective amount is in a range of about 2 $\mu g$ to 50 $\mu g$.

20. A kit for inducing cytotoxic T lymphocytes in a subject which comprises: a suitable amount of MHC class I restricted 8-12 amino acid antigenic peptide and a suitable adjuvant.

21. The method of claim 20, wherein the suitable adjuvant is "TITERMAX".

22. The method of claim 20, wherein the MHC class I restricted 8-12 amino acid antigenic peptide is selected from the group consisting of EVDPIGHLY, EADPTGHSY, EVVPISHLY, EIRSLYNPV, PLTSCNTSV, GYKDGNEYI, KYGVSVQDI, SIINFEKL, RGYVYQGL, FAPGNYPAL, VVYDFLKCL, SSIEFARL, ASNENMETM, GILGFVFPL, LLFGYPVYV, ILKEPVHGV, KLGEFYNQMM, IAGNSAYEYV, FLASDFFPSV (SEQ. ID. NOS. 1-19).

FIGURE 1A

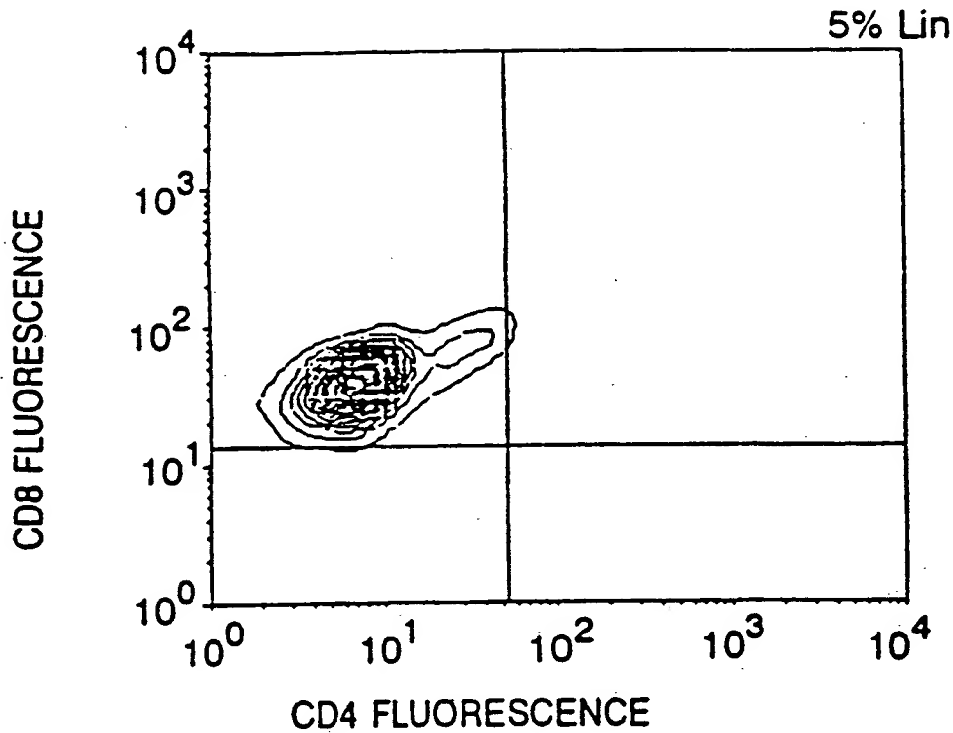

5% Lin
10^4
10^3
10^2
10^1
10^0
CD8 FLUORESCENCE
10^0
10^1
10^2
10^3
10^4
CD4 FLUORESCENCE

FIGURE 1B

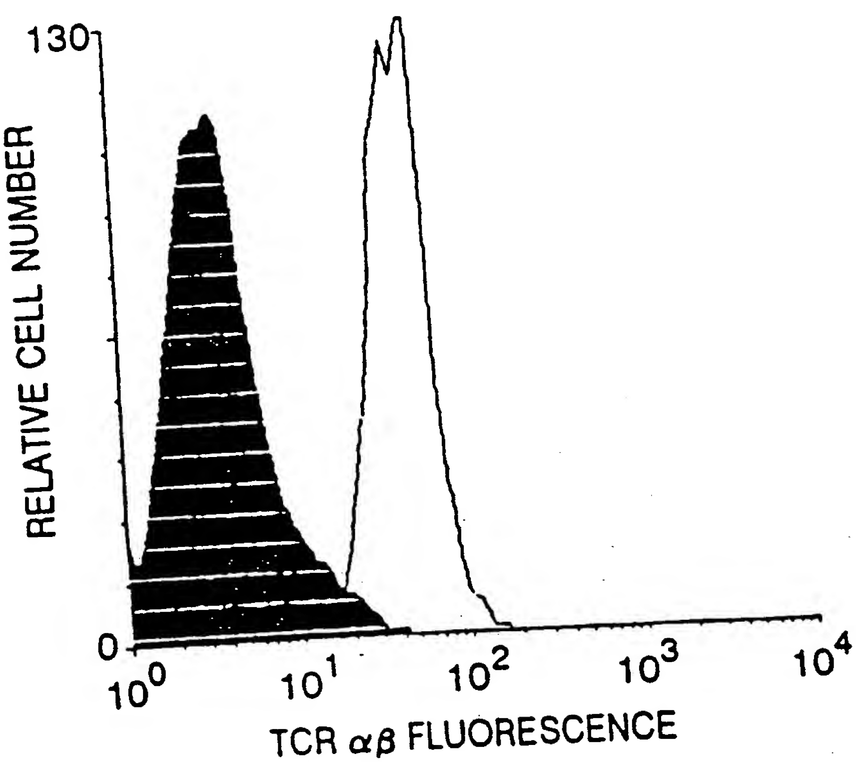

130
0
RELATIVE CELL NUMBER
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
TCR αβ FLUORESCENCE

FIGURE 2

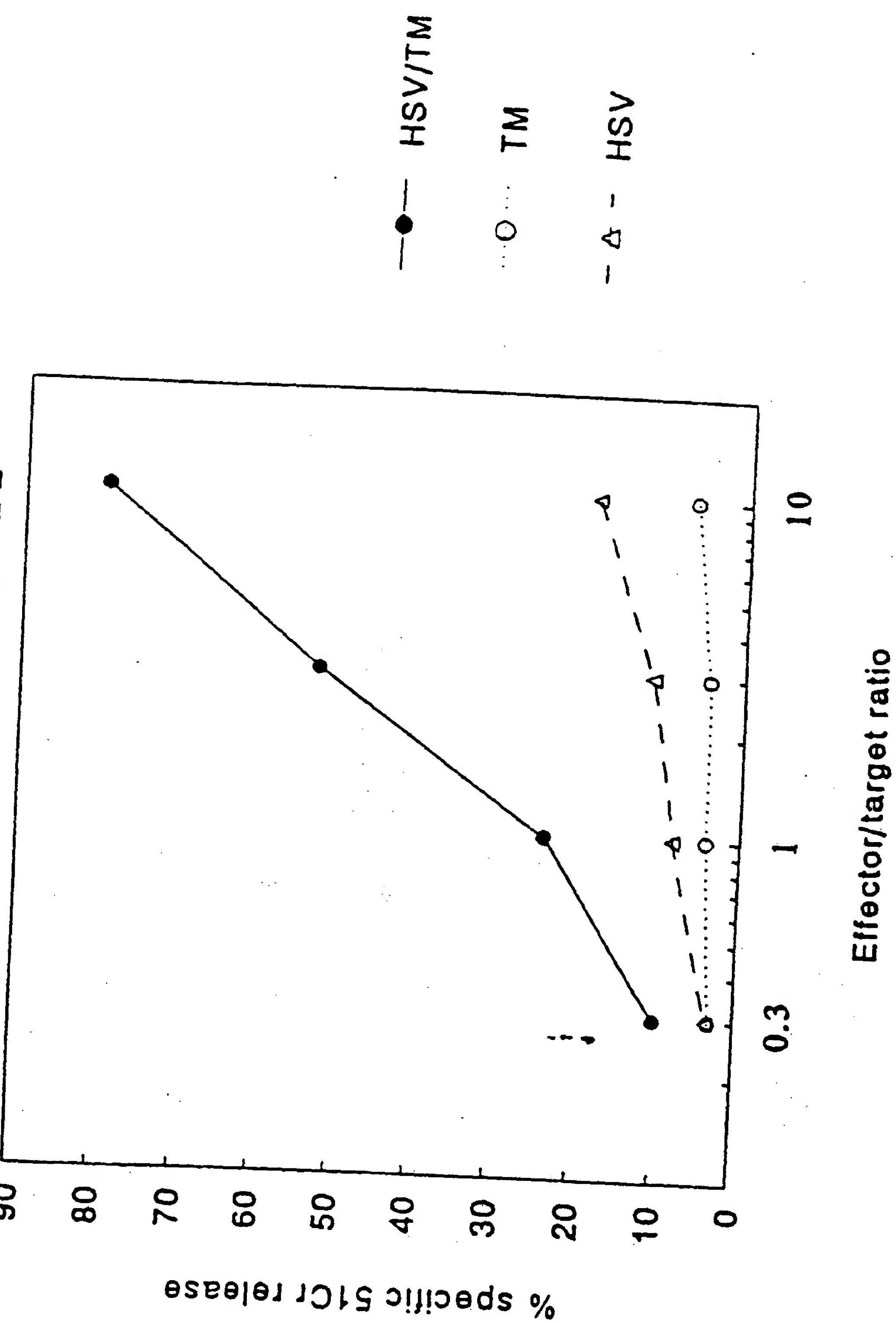

HSV/TM
TM
HSV
% specific 51Cr release
0
10
20
30
40
50
60
70
80
90
Effector/target ratio
0.3
1
10

FIGURE 3

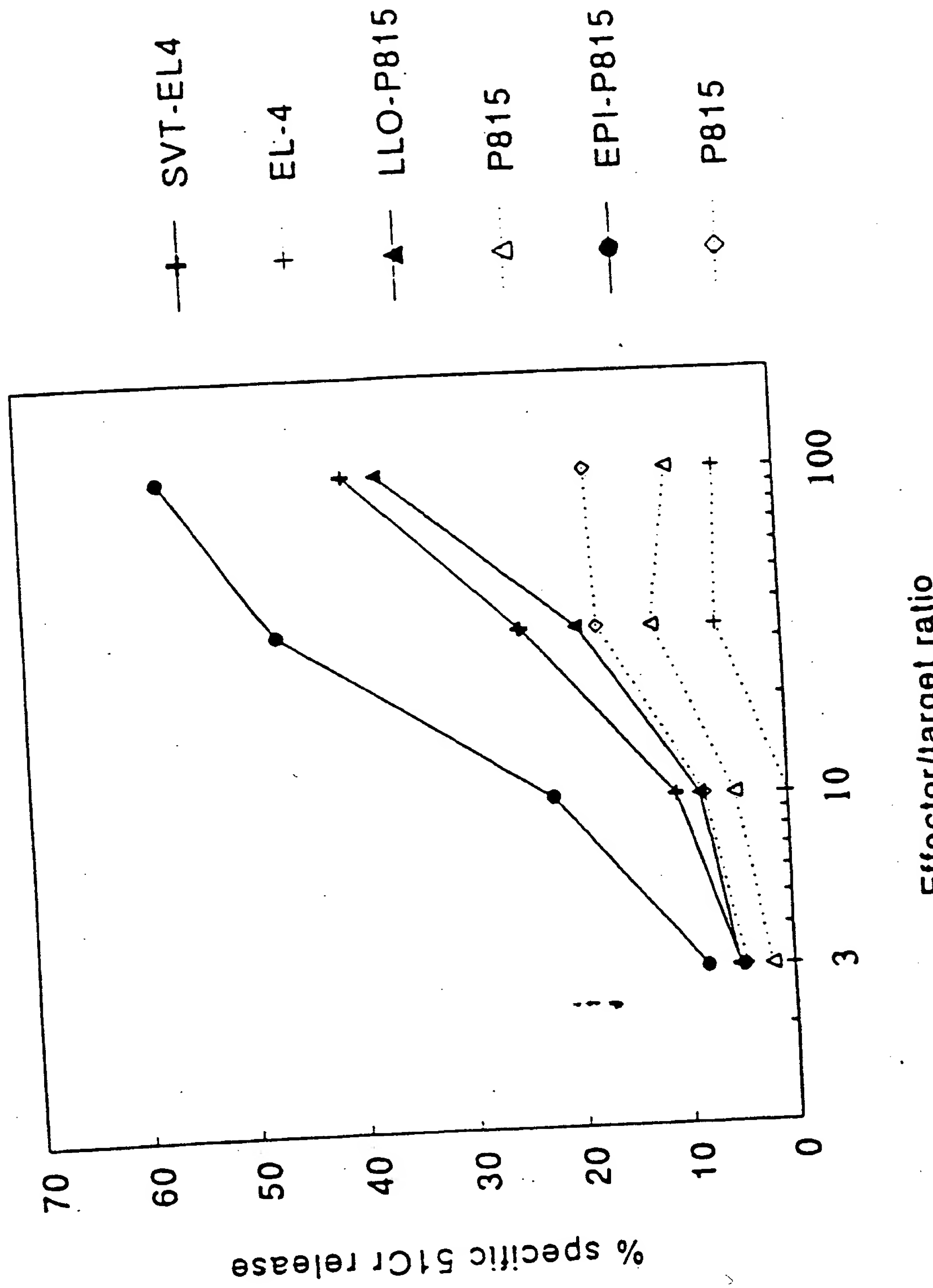

SVT-EL4
EL-4
LLO-P815
P815
EPI-P815
P815
% specific 51Cr release
0
10
20
30
40
50
60
70
3
10
100
Effector/target ratio

FIGURE 4A

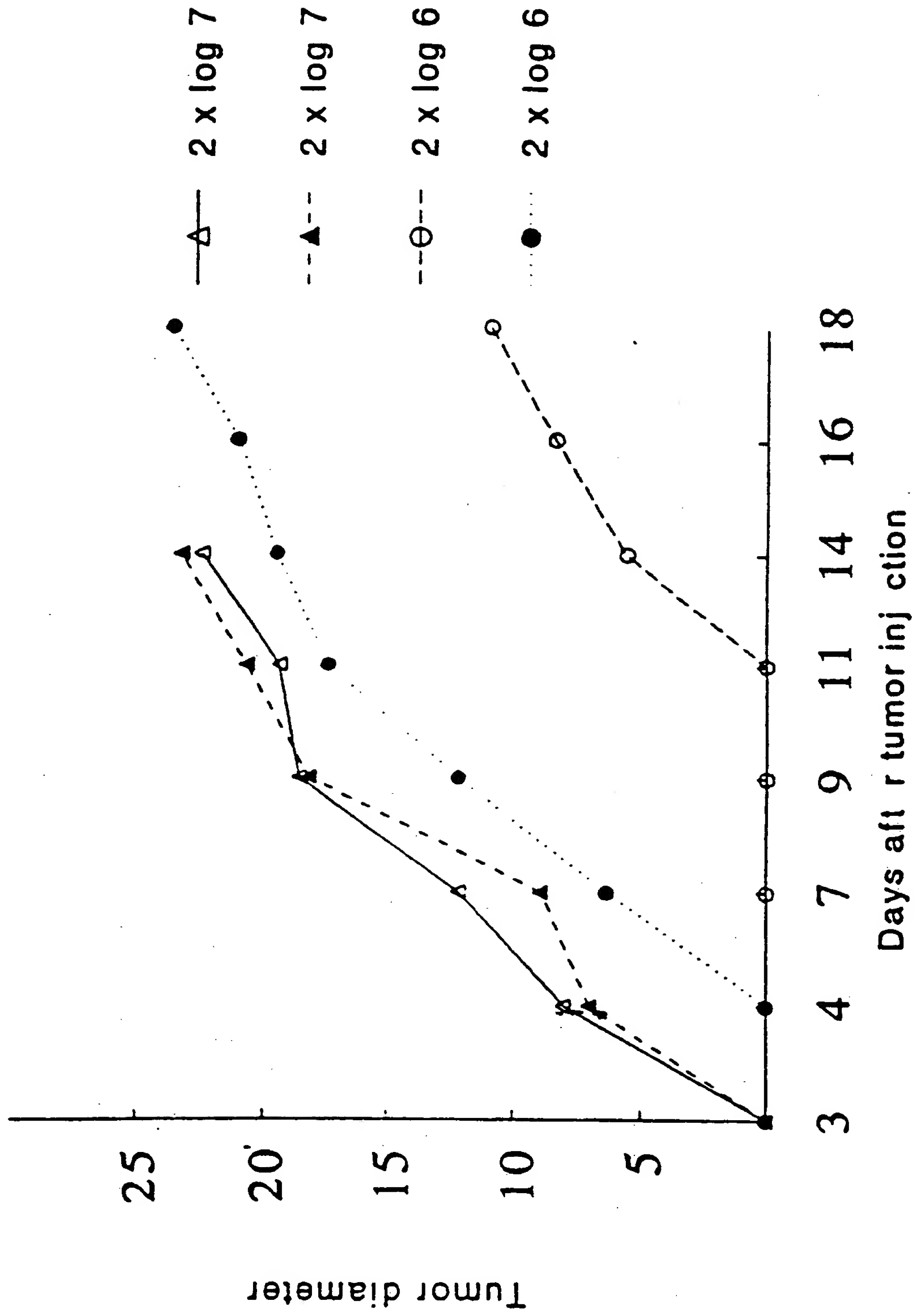

Tumor diameter
25
20
15
10
5
3
4
7
9
11
14
16
18
Days aft r tumor inj ction
2 x log 7
2 x log 7
2 x log 6
2 x log 6

FIGURE 4B

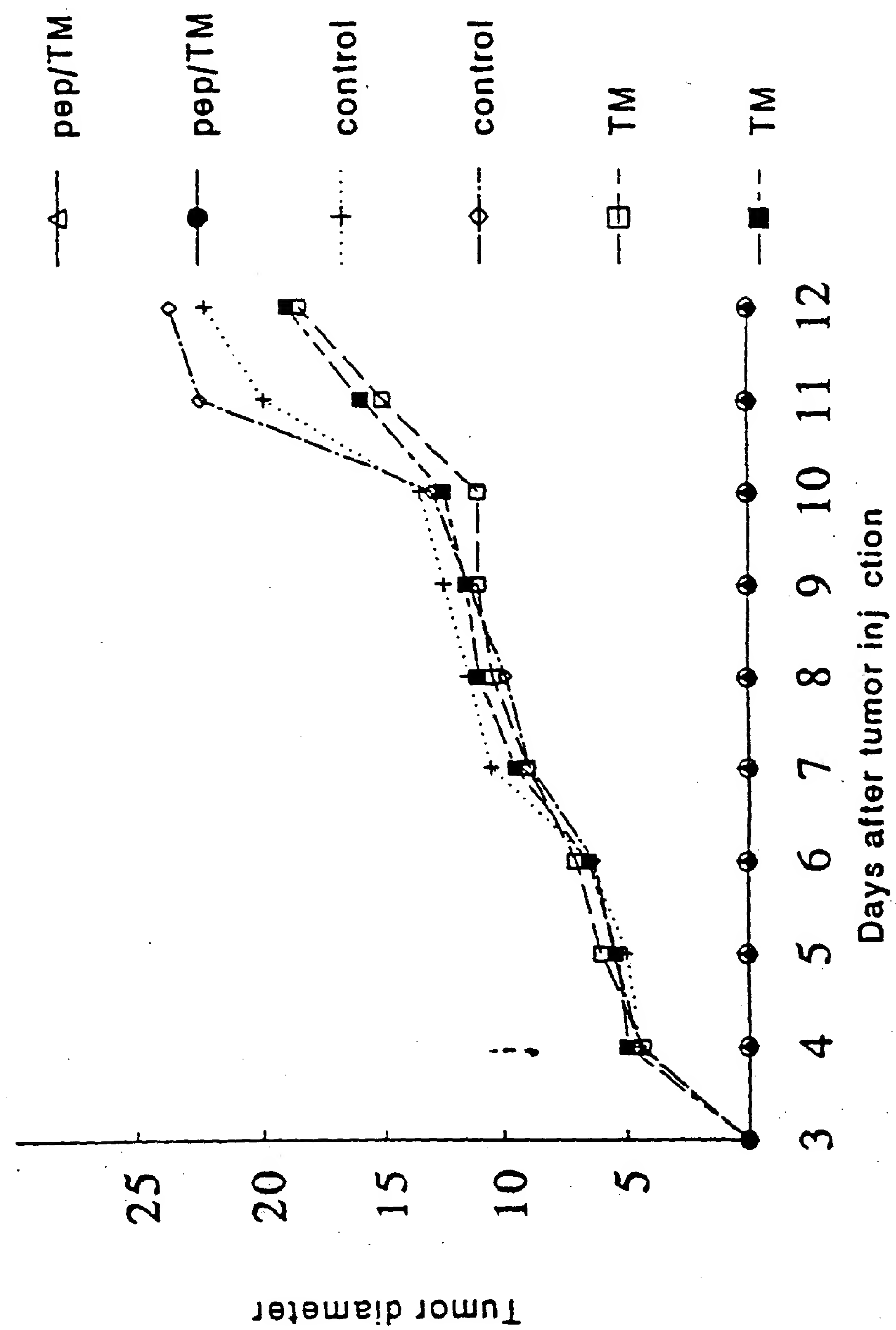

pep/TM
pep/TM
pep/TM
control
control
TM
TM
25
20
15
10
5
Tumor diameter
3
4
5
6
7
8
9
10
11
12
Days after tumor inj ction

FIGURE 5

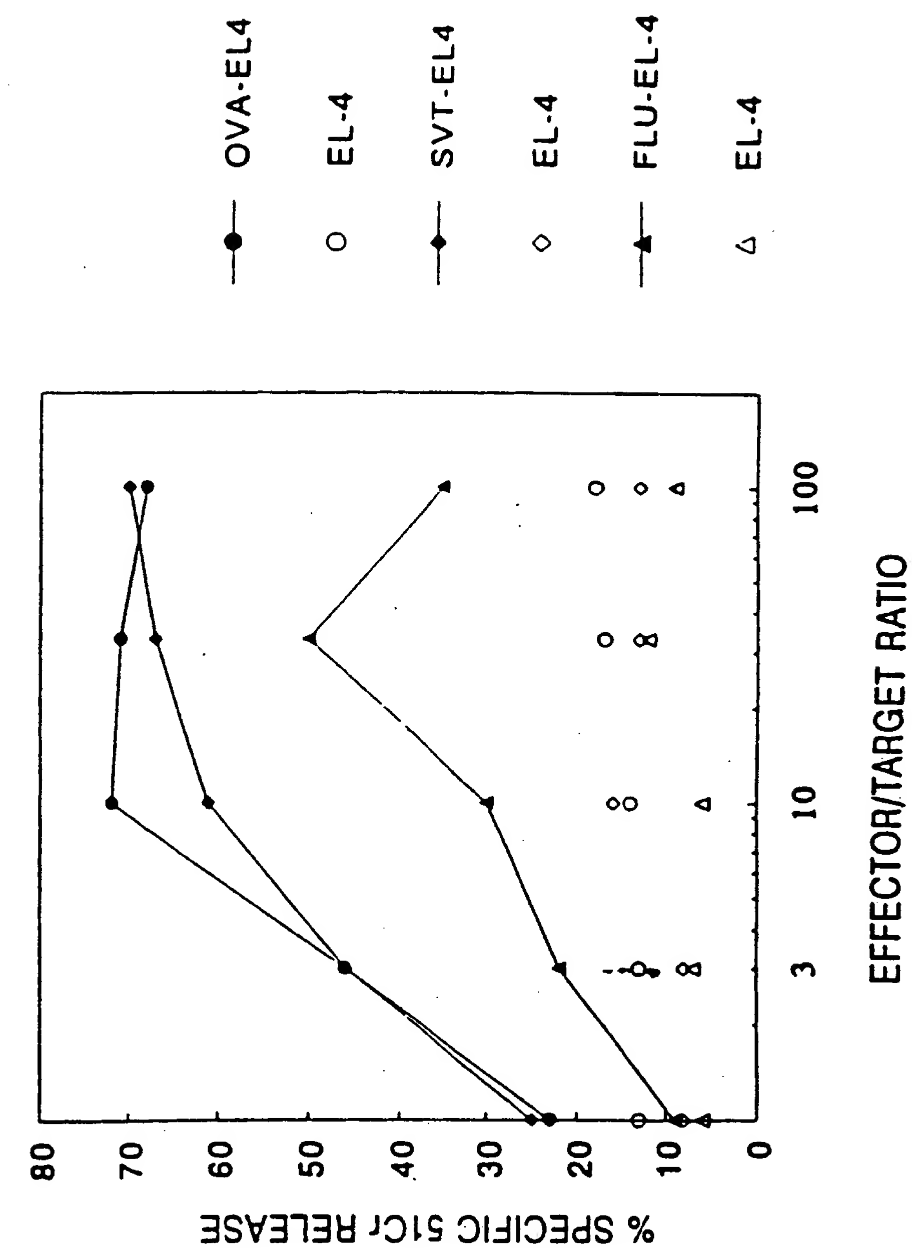
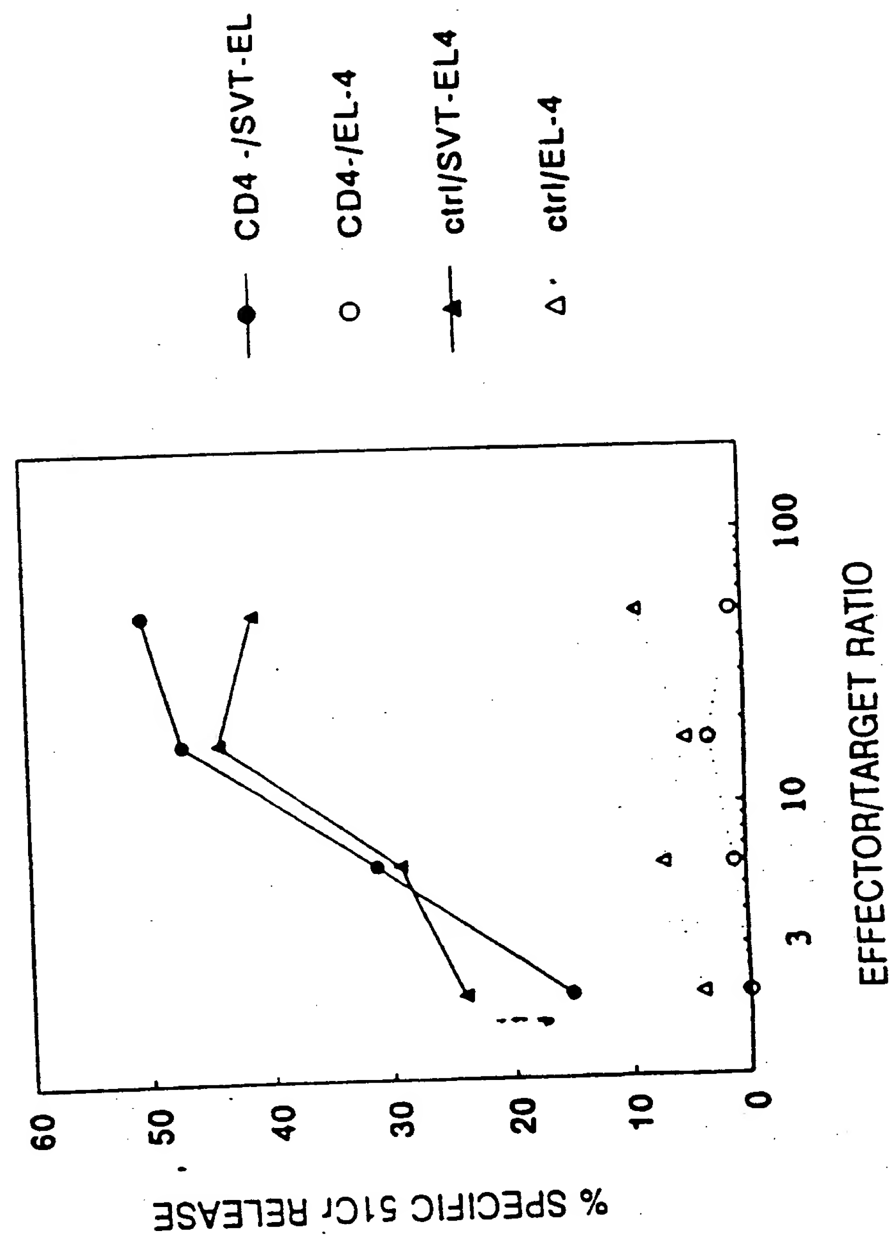

OVA-EL4
EL-4
SVT-EL4
EL-4
FLU-EL-4
EL-4
% SPECIFIC 51Cr RELEASE
0
10
20
30
40
50
60
70
80
3
10
100
EFFECTOR/TARGET RATIO

FIGURE 6


CD4 -/SVT-EL
CD4-/EL-4
ctrl/SVT-EL4
ctrl/EL-4
% SPECIFIC 51Cr RELEASE
60
50
40
30
20
10
0
3
10
100
EFFECTOR/TARGET RATIO

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04975

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 39/00, 39/385, 39/39; C07K 14/74
US CL : 424/184.1, 185.1, 277.1, 278.1; 530/300, 350, 395
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 185.1, 277.1, 278.1; 530/300, 350, 395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Automated Patent System (APS) and DIALOG (file BIOCHEM) databases. Key words: MHC Class I, tumor, cancer, peptide.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | US, A, 4,478,823 (SANDERSON) 23 October 1984, see the claims. | 1-22 |
| A | US, A, 5,130,297 (SHARMA ET AL.) 14 July 1992, see entire document. | 1-22 |
| Y | W. F. Paul, "Fundamental Immunology", 3rd edition, published by Raven Press (N.Y.), pages 615-628, see entire section. | 1-22 |
| Y | Cancer Research, vol. 53, issued 01 January 1993, Zakut et al., "Differential expression of MAGE-1, -2, and -3 messenger RNA in transformed and normal human cell lines", pages 5-8, see entire article. | 1-22 |
| Y | US, A, 5,200,320 (SETTE ET AL.) 06 April 1993, see entire document. | 1-22 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search: 17 JULY 1995

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